DIURNAL EXPRESSION OF MUSCARINIC AND DOPAMINERGIC RECEPTORS IN THE EYE OF ZEBRAFISH

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Thank you to my family and friends for their unwavering support. From the bottom of my heart, THANK YOU to my other half, Jason and my little one, Payton.

This thesis may have never been if it were not for the guidance of Dr. Koke, who introduced me to cell biology and piqued my interest in the workings of the small and invisible from the beginning, and Dr. García with enough intellect, understanding and patience to go around.

And a HUGE thank you to my labmates, especially Katie Saul and Mya Patel, who were there through the awesome and exciting times, the delirium, and the frustration. And thanks to Luis Neve for help with dissections and intriguing conversation.

To the hurdles we jump, the mountains we climb...here is my success in the midst of chaos.

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ABSTRACT

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Diurnal rhythms allow organisms to synchronize physiological functions, such as feeding habits, with environmental cues such as light and temperature. Much is known about the diurnal and/or circadian regulation of neurotransmitters, but there is little to no knowledge of the diurnal or circadian regulation of the G-protein coupled receptors (GPCRs) on which they act. This study sought to determine whether there was a diurnal pattern of gene expression for muscarinic and dopaminergic GPCRs and the GPCR

regulator, arrestin. Quantitative reverse transriptase polymerase chain reaction was employed on mRNA extracted from whole eye of zebrafish at four time points - dawn, midday, dusk, and midnight - indicated a diurnal pattern of gene expression for 6 genes out of 11 analyzed, arrestin, D2A, D4A, D4C, M_2, and M_5. Reverse transcriptase-PCR of mRNA extracted from different parts of the eye was used to localize expression of the statistically significantly differentially regulated genes. These results indicate expression of all receptor subtypes in the retina and RPE. Some of the receptors were found in other parts of the eye depending upon time of day, and arrestin was found in all parts at all time points. Immunohistochemical analysis of the muscarinic M_3 receptor and arrestin revealed the protein to be localized to different layers within the retina depending upon the time of day. Taken together, these results suggest that dopaminergic and muscarinic receptors, which have been implicated by others in the regulation of various circadian and diurnal processes in the eye, are themselves, along with arrestin, subject to diurnal regulation.

CHAPTER I

INTRODUCTION

Diurnal and circadian rhythms function to coordinate the physiology of an organism with the daily cycle of changes in its environment (for review see Edery, 2000). In humans, the suprachiasmatic nuclei (SCN) serves as the master clock, synchronizing the peripheral clocks with a Zeitgeber (ZT) (Klein et al., 1991). The eye helps to set this clock by encoding light that enters through the eye as action potentials, information that is relayed to the SCN. The master clock then influences the neuro-endocrine system to synchronize physiological functions in coordination with the environment. In this way, diurnal patterns of physiological changes are established, and a subset of these, circadian rhythms, persist at least temporarily in the absence of the light cycle, that is, in persistent dark or persistent light. Although there are peripheral tissues that have phototranducing elements, the eye is the primary light receptor for the body; thus the study of diurnal and circadian rhythms in the eye is necessary for understanding the overall coordination of circadian rhythms.

In the retina of fishes, it is known that neurotransmitters undergo diurnal or circadian patterns of synthesis and release for control of light and dark adaptive processes, such as retinomotor movements of the photoreceptors and melanin pigment granules in the retinal pigment epithelium (RPE). Fish, lacking a constricting pupil, use retinomotor movements involving the photoreceptors and RPE to regulate the amount of

light impinging on the photoreceptors RPE is a single layer of cuboidal cells that contain membrane bound melanin pigment granules and have long apical projections that intertwine with the rods and cones (see Zinn and Marmor, 1979; Ali and Anotil, 1976). During the day when there is a high intensity of light, the pigment granules disperse into the projections while the rods elongate and cones contract; these movements protect the rods from photobleaching (Douglas, 1982). During the night, these movements are reversed. Studies in zebrafish have been conducted that suggest these diurnal changes in retinomotor movements, particularly in the photoreceptors, are regulated by a circadian oscillator (Menger et al., 2005) since they persist in conditions of constant darkness.

Two neurotransmitters that are most well studied in the context of diurnal and circadian rhythms are dopamine and melatonin. In the Midas cichlid, *Cichlasoma citrinellum,* tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis, reaches its peak activity at midnight and declines toward dawn (McCormack and Burnside, 1993). Dopamine release in goldfish is greater at subjective midday (ZT=6 hours) versus subjective midnight (ZT=18 hours); dopamine at other time points has not been examined in goldfish (Ribelayaga et al. 2002). Dopamine has been well established as a light signal within the retina of fishes, causing the light adaptive retinomotor movements described above. In green sunfish, *Lepomis cyanellus*, dopamine causes the cones to contract and pigment granules to disperse into the apical projections of the RPE (Dearry and Burnside 1986a). Fish, like humans, have two major classes of dopamine receptors: D1-like and D2-like (Missale et al. 1998). The light-adaptive effects of dopamine observed in green sunfish were implemented through a D2-like receptor (Dearry and Burnside 1986a; Dearry et al. 1990). In humans, the D2-like receptors

include D2, D3 and D4, but fish have not only 5 subtypes (D1-D5) as is the case in humans but also subtypes of subtypes (D1, D2A, D2B, D2C, D4A, D4B, D4C, D5).

In the teleost eye dopamine has a variety of functions in addition to the induction of light adaptive retinomotor movements in photoreceptor and retinal pigment epithelium discussed above (Dearry and Burnside 1989; Dearry et al. 1990). These functions include modulating potassium currents in bipolar cells (Yu and Li 2005) and mediating rod and cone input to horizontal cells, inputs which are rod-dominated at night, but cone-dominated during the day (Ribelyaga et al. 2002). These observations taken together with the changes in tyrosine hydroxylase activity suggest that dopamine needed for light adaptation may be synthesized in preparation for dawn.

While dopamine is generally regarded as a day/night signal, melatonin functions in many animals as a night/dark signal. Arylalkylamine N-acetyltransferase (AANAT) is the rate-limiting enzyme in the melatonin biosynthetic pathway, and has been shown by a number of investigators to have increased activity at midnight (Olcese and Moller 1989; Zurwaska and Nowak 1992; Applebaum et al. 2006) along with the release of melatonin (Klein et al. 1997). It is important to point out the interplay of dopamine and melatonin for the induction of light adaptive and dark adaptive retinomotor movements. The increase in melatonin release at night inhibits the release of dopamine (Zawilska et al. 2003), and the increase of dopamine regulates the synthesis of melatonin by inhibiting the increase of AANAT at night (Besharse et al., 1984). In mice, melatonin has been shown to be released from photoreceptors, reaching peak levels at midnight (Klein et al. 1997). The complex interplay of melatonin and dopamine controls light and dark adaptation

within the retina. The synthesis and release of other neurotransmitters involved in retinomotor movements, such as acetylcholine, have not been studied extensively.

Previous research has shown that carbachol, a cholinergic agonist, causes pigment granule dispersion in isolated teleost RPE (García et al. 1998). To further elucidate the role of muscarinic receptors in this process, González et al. (2004) and Phatarpekar et al. (2005), used an assortment of receptor agonists and antagonists to determine the specific muscarinic receptor subtype involved in pigment granule dispersion in bluegill RPE. They discovered that the antagonists and agonists that targeted M_{odd} receptors were successful at inhibiting carbachol-induced dispersion or initiating dispersion, respectively. Like dopamine receptors, the situation with muscarinic receptor is complex due to number of subtypes, and previous research has shown that zebrafish have more than the five subtypes that exist in humans (Nuckels 2005). Additionally, Phatarpekar et al. (2005) used RT-PCR to show the presence of the M_5muscarinic receptor in bluegill retina and RPE and the absence of the M_2receptor in the RPE but not retina. From these studies it is thought that a M_{odd} receptor subtype is responsible for pigment granule dispersion, and it is less likely that a M_{even} subtype is involved.

Diurnal and circadian fluctuations in neurotransmitter synthesis and release have been established without consideration of the expression of their cognate receptors. It may be that the cognate receptors also undergo a diurnal pattern of expression, both at the transcriptional and translational levels. There are many receptors that respond to the same ligand with varying affinities and different downstream signaling pathways, and the receptor present determines the physiological response. One GPCR for which circadian regulation has been established is zebrafish rhodopsin. Yu et al. (2006) looked at

rhodopsin expression using qRT-PCR in a transgenic zebrafish with short half-life green fluorescent protein (GFP) under transcriptional control of the rhodopsin promoter. They found that the expression levels of rhodopsin mRNA changed in a rhythmic circadian fashion, and light set this rhythm by decreasing rhodopsin expression. They also found that expression changed upon the addition of dopamine receptor agonist and antagonists, and that entrainment of rhodopsin circadian rhythms by light are mediated by dopamine via a D2-like receptor (Yu et al. 2006). The research of Yu et al. (2006) establishes a good argument for looking at the diurnal regulation of other receptors, such as dopamine and muscarinic receptors, at the transcriptional level in the retina to elucidate their role in retinal physiology as well as in the coordination of physiology of the organism.

Since both muscarinic and dopaminergic receptors are G-protein coupled receptors, the diurnal rhythm of arrestin expression are of interest. There are several ways a GPCR-dependent signal is attenuated, one of which is by the protein arrestin. Nonvisual arrestins, β-arrestin-1 and β-arrestin-2, bind after a receptor has been phosphorylated by a G-protein coupled receptor kinase (GRK) (Krupnick and Benovi 1998). The receptor is then either endocytosed or sequestered for later resensitization (Zhang et al. 1997). Rod arrestin (Arr-1) regulates rhodopsin specifically by binding to the phosphorylated receptor and thus attenuation of signal (Baylor and Burns 1998; Wilden et al. 1986a), but little is known about the role of cone arrestin (Arr-3) in light adaptation of cone photoreceptors in vivo and in zebrafish. It is thought that cone arrestin is involved in responding to the phosphorylation of opsins much like the relationship between rod arrestin and rhodopsin (Sutton et al. 2005).

The diurnal pattern of expression of muscarinic receptors, dopaminergic receptors, and arrestin may reveal important insights into the retinal physiology of the zebrafish. This study sought to identify a diurnal pattern of gene expression and to relate it to retinal physiology. Since retinomotor movements have been to shown to be a diurnal process I hypothesize that transcription of receptor mRNA and subsequent protein synthesis involved in these processes also undergo diurnal regulation. Quantitative reverse transcriptase PCR (qRT-PCR) was employed to identify changes in mRNA expression and reverse transcriptase PCR (RT-PCR) to identify the part of the eye expressing particular transcripts. Changes at the protein level were also evaluated using immunohistochemical techniques and western blot analysis.

CHAPTER II

MATERIALS AND METHODS

Fish Maintenance

Wild type zebrafish, *Danio rerio*, (ZDR) were obtained from Aquatica Tropicals (San Marcos, FL). Fish were maintained on a 12 hour light, 12 hour dark cycle for at least 2 weeks before use. Fish were cared for and sacrificed following Texas State IACUC protocols069744F82 and 069744F82-0927_0928_30.

RNA Extraction

RNA was extracted using the phenol-chloroform method (Chomczynski and Sacchi 1987). Eye tissue was extracted and 1 ml of TRI reagent (Ambion, Austin, TX) was added. Samples were homogenized and incubated at room temperature for 5 minutes. Samples were centrifuged using the Eppendorf 5415R tabletop centrifuge for 10 minutes at 14,000 RPM to remove debris, and the supernatant was transferred to a new 1.5 ml microcentrifuge tube. Tubes were incubated at room temperature for 15 minutes with 200 µl of chloroform (Sigma-Aldrich, St. Louis, MO). After incubation, samples were centrifuged for 15 minutes at 14,000 RPM, the aqueous phase was kept, and the organic phase containing protein and DNA was discarded. To precipitate the RNA, 500µlisopropanol was added along with 3.5 µl of 15mg/ml Glyco Blue to aid in precipitation and add color to the RNA pellet. Samples were incubated at -20°C for

20 minutes. After incubation, samples were spun 8 minutes at 14,000 RPM and the supernatant was discarded. RNA pellets were washed with 1ml 75% ethanol for 5 minutes at 10,000 RPM. Remaining ethanol was removed using a fine tip pipette, and samples were allowed to air dry completely before dissolving in 100 µl RNase free water. Samples were subsequently cleaned using the Qiagen RNeasy Mini Kit (Valencia, CA) and quantified using the Nucleic Acids function on a Nanodrop Spectrophotometer (Thermo Scientific, Willmington, DE).

cDNA Synthesis

Single stranded complementary DNA (cDNA) was synthesized using the Ambion Retroscript RT kit (Austin, TX) according to manufacturer's instructions with 200ngtotal RNA as starting material. All samples were incubated at 44°C for 1 hour and 92°C for 10 minutes using the Eppendorf Realplex (Hamburg, Germany) and stored at -20°C until use. The cDNA was subsequently diluted by adding 200 µl RNase-free water to each sample tube. The samples were diluted further 1:10 to be sure that there was enough cDNA for all assays.

Real-Time Polymerase Chain Reaction (qRT-PCR)

Second strand synthesis was performed using the SYBR Green ER Universal Mastermix (Invitrogen, Location) on 12ngof cDNA The following program was performed using the Eppendorf Realplex (Hamburg, Germany)· 50°C for 2 minutes, 95°C for 10 minutes and 50 cycles of 95°C for 15 seconds, 60°C for 15 seconds, and 68°C for15 seconds. Results were analyzed using the ΔΔCt method (Livak and Schmittgen, 2001). B-actin was used as the reference gene and was shown not to fluctuate in this model using the reference gene analysis equation from the ΔΔCt method. Results of the

ΔΔCt method are presented on bar graphs with relative Ct on the y-axis and time of day on the x-axis.

Tissue and Slide Preparation

Fish were sacrificed at dawn, midday, dusk, and midnight. Whole eyes were harvested and placed in 4% paraformaldehyde overnight at room temperature. Subsequently, eyes were cryoprotected in 30% sucrose overnight. Eyes were then embedded in Tissue-Tek OCT, sectioned at 20μm using a Zeiss Microm cryostat (New York, NY). Sections were collected on 22 mm^2coverslips coated with poly-l-lysine (Sigma-Aldrich) and stored at 4°C until use.

Immunohistochemistry (IHC)

IHC was performed to localize M_3receptor and cone arrestin expression in the retina. Sections were washed 5 minutes in 0.2% PBST (pH 7.4, 137mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4(PBS), 0.2% Tween) before adding blocking solution (10% non-fat dry milk in PBS) for 1 hour. Sections were then washed with PBST 3 times for 5 minutes each before the addition of primary antibody. Polyclonal rabbit anti-human M_3 I_2 loop (Abcam 13063) or chicken anti-cone arrestin (ProSci Inc., Poway, CA) was diluted 1:150 in PBS and added to sections overnight at 4°C. After incubation in primary antibody, sections were washed 3 times for 15 minutes each with PBST. Secondary antibody goat anti-rabbit conjugated to AlexaFluor 488 (Invitrogen) diluted 1:200 or rabbit anti-chicken conjugated to TRITC (ProSci, Inc.) diluted 1:300 was added to the sections for 2 hours at room temperature. Sections were then washed with PBST 3 times for 10 minutes each. DAPI was added for 5 minutes and sections were washed 3 times for 10 minutes each with PBS. Coverslips were mounted on slides with

90% glycerol. The coverslips were viewed using the FV1000 Confocal Laser Microscope; all images were acquired using a 20X lens. Gain and dynamic settings were calibrated using the experimental and kept the same for controls and all subsequent other time points.

Protein Extraction

Eyes were removed from zebrafish at dawn, midday, dusk, and midnight and placed in ~80µl extraction buffer (0.1% Triton X-100, 1 mM EDTA, and 35 µg/ml PMSF in PBS). Samples were then homogenized using a mini-pestle in a 1.5 ml centrifuge tube, and homogenates were allowed to sit on ice for 30 minutes. After 30 minutes samples were centrifuged using the Eppendorf 5415R at 14,000 RPM. The supernatant was transferred to a1.5 ml centrifuge tube and stored at -20°C until use. Protein concentrations in the homogenates were determined using the Nanodrop Spectrophotometer A280/260 assay.

Western Blot Analysis

Protein supernatants were combined with 5X sample buffer (50 mM Tris-HCl, 25% glycerol, 2% SDS, 5% 2-mercaptoethanol and 0.1% bromophenol blue). Samples were then boiled for 10 minutes and centrifuged at top speed in a tabletop centrifuge to remove debris. Approximately 14µg of protein, estimated by the A280/260 assay, were loaded onto a 12% separating gel that was prepared with a 4% stacking gel along with Precision Plus Protein standard (Bio-Rad, Hercules, CA. catalog number 161-0373). Electrophoresis was performed for 30 minutes at 200 volts by SDS-PAGE using a Bio-Rad (Hercules, California) Mini-Protean II system. One gel was stained using Coomassie blue gel stain solution (50%methanol, 5% glacial acetic acid, 1.17 mM Coomassie Blue-

G 250) for 15 minutes and subsequently destained with Coomassie destain (10% methanol and 10% glacial acetic acid) overnight. Proteins on the sister gel were transferred to nitrocellulose (Biorad, Ausitn, TX) at 100 volts for one hour. After the transfer, the nitrocellulose was washed in PBS 3 times for 5 minutes each and incubated in blocking solution (10% non-fat dry milk in 1X PBS) 45 minutes. Primary antibody, polyclonal rabbit anti-human M_3 I_2 loop (Abcam 13063), was prepared at a 1:900 dilution in PBS, and the nitrocellulose was incubated in antibody-containing solution overnight at 4°C. The nitrocellulose was then washed 3 times for 5 minutes each in PBS. Next, the nitrocellulose was incubated for one hour in secondary antibody, goat anti rabbit IgG conjugated to alkaline phosphatase (Invitrogen), that was prepared at a 1:30,000 dilution in blocking solution. Detection of bands was achieved by dissolving a Sigma-Fast NBT/BCIP tablet (Sigma-Aldrich) in 10 ml of deionized water and incubating the nitrocellulose for 10 minutes in the resulting solution. The reaction was stopped by rinsing the nitrocellulose with deionized water.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The polymerase chain reaction was performed on cDNA synthesized from RNA extracted from different parts of the eye (sclera and choroid, retina and RPE, and cornea and lens) to localize the expression of genes analyzed by qRT-PCR. RNA was extracted and cDNA synthesized as described above except 200 ng cDNA was synthesized and not diluted any further. The reaction was carried out as follows: 2 µl single stranded cDNA, 0.5 µl Taq Polymerase (Gentech, Arcade, NY), 5 µl 10X Reaction Buffer (Gentech), 1 µl of 100µM forward primer, 1 µl of 100 µM reverse primer, and 1 µl of 10 mM dNTPs (Promega, Madison, WI). The following cycler program was performed using an

Eppendorf Realplex (Hamburg, Germany): 94°C for 3 minutes then 50 cycles of: 94°Cfor 15 seconds, 60°C for 15 seconds, and 72°C for 20 seconds, and extension at 72°C for 2 minutes.

Products from PCR were separated on an agarose gel to determine whether the product size matched the predicted size. A 3% agarose gel was made using TAE Buffer (0.04 Tris-acetate and 0.001 EDTA) and 20 µl ethidium bromide. PCR products were run along with Benchtop Standard (Promega) for 2 hours at 90 volts. The gels were imaged using a Canon Powershot A260 with ZoomBrowser EX Software.

Statistical Analysis

Relative cycle threshold (Ct) values obtained from qRT-PCR were analyzed using the StatPlus program (AnalystSoft, StatPlus: mac - statistical analysis program for Mac OS. Version 2008. See http://www.analystsoft.com/en/). Cycle threshold (Ct) is the point at which fluorescence levels from the binding of SYBR-Green to double stranded DNA (product) reaches above background fluorescence levels. The relative Ct values are calculated by subtracting Ct values of a reference gene from the Ct value of the gene of interest(Ct of gene of interest - Ct of ß-actin). A one-way analysis of variance (ANOVA) with post hoc Tukey HSD.A P value of$\leq$ 0.05 was taken to indicate significant differences.

CHAPTER III

RESULTS

Diurnal Expression of Dopaminergic Receptor mRNA in the Eye

Quantitative reverse transcriptase PCR (qRT-PCR) was employed on RNA extracted at dawn, midday, dusk and midnight to determine if there was a diurnal pattern of expression of dopaminergic receptors. Both D2A and D4A mRNA expression reached their peak at midnight. However, D2A mRNA expression at dawn was near its highest levels and reached its nadir at dusk while D4A mRNA was at its nadir at dawn and reached its highest levels at midnight. D4C mRNA reached its peak expression at midday and its lowest levels at dusk and midnight (Figure 1). The results also indicate that D4A may be the predominant subtype in the eye. The dopamine receptors were also localized to different parts of the eye using RT-PCR with the exception of D2A. Expression of D4A mRNA was localized to the retina and RPE at all time points (Figure 2). It was also localized to the sclera and choroid at midday and dawn and lens and cornea at dusk. D4C mRNA expression was found in all layers of the retina at all time points (Figure 3).

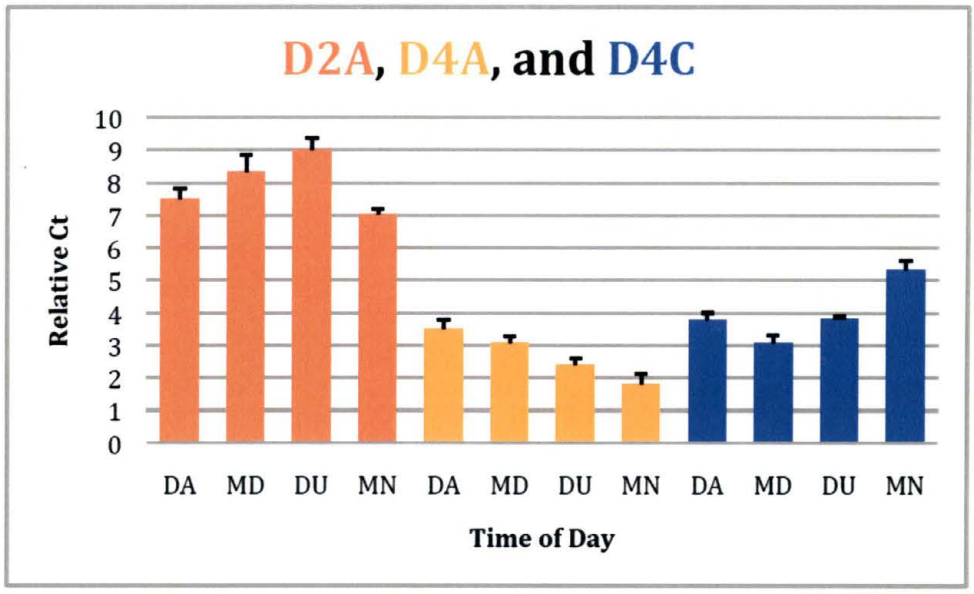

Figure 1. qRT-PCR results: Dopaminergic receptors. Eyes were collected at the four time points: dawn, midday, dusk, and midnight. RNA was extracted and subsequently subjected to cDNA synthesis and qRT-PCR. The bar graph shows the relative Ct values (Ct of gene of interest - Ct of ß-actin) vs. time of day (dawn, midday, dusk, and midnight). The lower the mean relative Ct, the higher greater the starting amount of mRNA for the gene of interest.

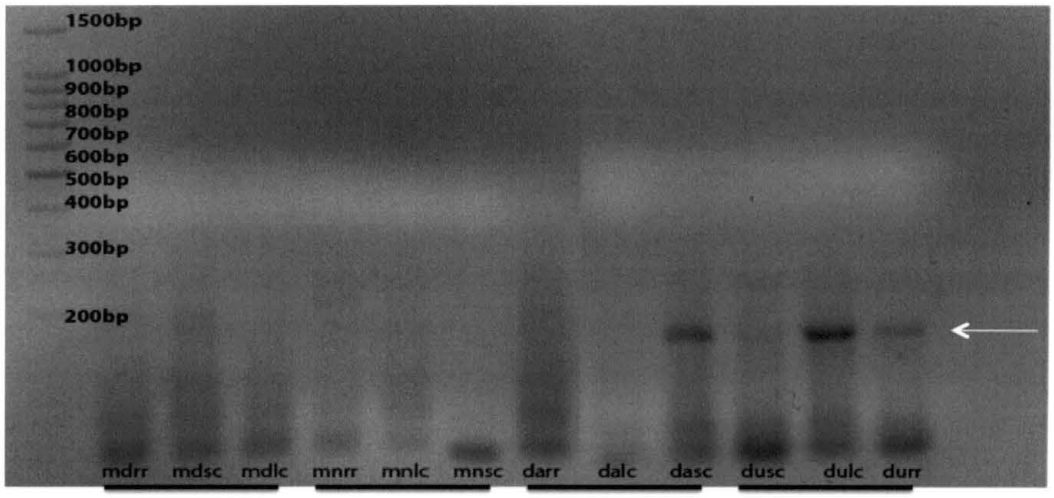

Figure 2. Localization of D4A receptor to different parts of the eye. The expected product size of 191 bp was generated from RNA extracted from the retina and RPE at all time points. The same product was also found from sclera and choroid at dawn as well as dusk and the lens and cornea at dusk. The lanes are labeled as follows: first two letters are the time of day, da=dawn, md=midday, du=dusk and mn=midnight and the last two letters are the parts of the eye from where the tissue was taken; rr=retina and RPE, lc=lens and cornea, and sc=sclera and choroid.

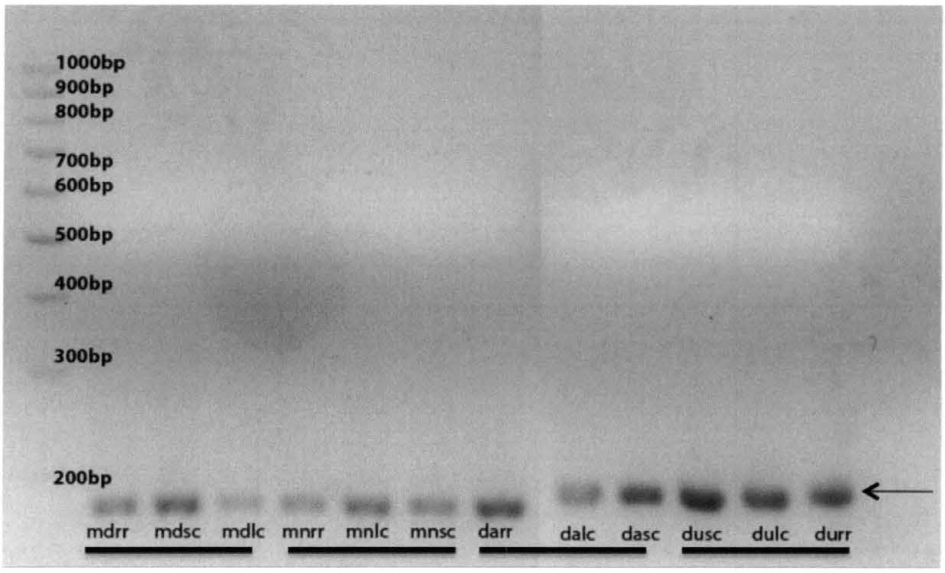

Figure 3. Localization of D4C receptor to different parts of the eye. The expected product of 175 bp was generated from RNA obtained from all parts of the eye at all time points.

Diurnal Expression of Muscarinic Receptor mRNA in the Eye

Quantitative RT-PCR was employed on RNA extracted at the above mentioned time points. $M_2$and M_5receptor mRNA both reach their peak expression at midnight. The expression of M_2 mRNA follows a trend beginning with an increase in expression at dawn and leveling off at midday and dusk. At midnight, the expression increases again to a level that is significantly different from all other time points (Figure 4). The results also indicate that the M_2 subtype is the predominant muscarinic receptor subtype in the eye. M_5 has high expression at dawn but not as much as midnight (Figure 4). Expression of mRNA was found in different parts of the eye at the various time points. M_2 mRNA was found in the retina and RPE only at all time points with the exception of dawn and dusk, where it was expressed in sclera and choroid. It was also expressed in the lens and cornea at dusk (Figure 5). M_5 mRNA was found in all parts of the eye except the lens and cornea at dawn (Figure 6).

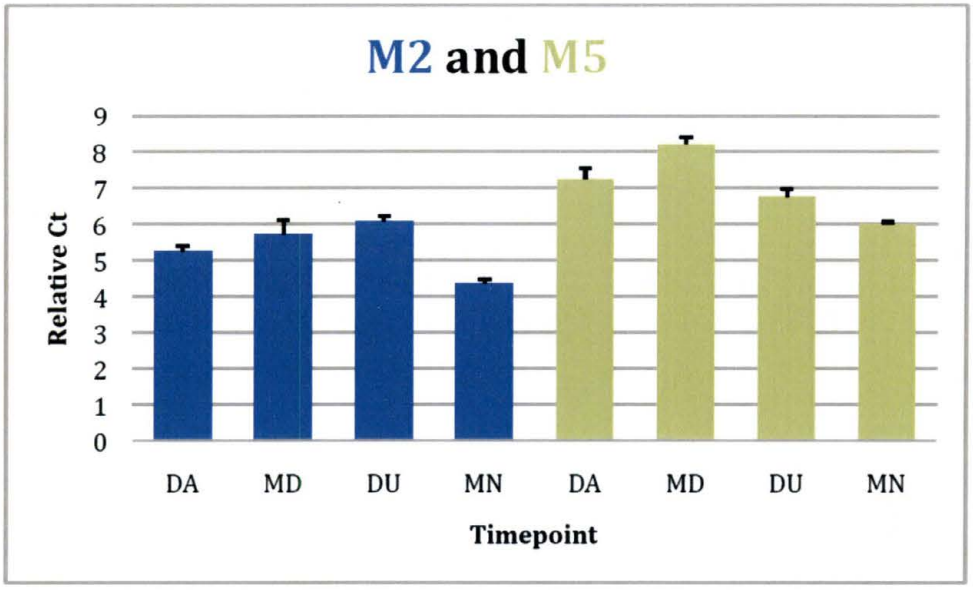

Figure 4. qRT-PCR results: Muscarinic receptors. Eyes were collected at the four time points: dawn, midday, dusk, and midnight. RNA was extracted and subsequently subjected to cDNA synthesis and qRT-PCR. The bar graph shows the relative Ct values (Ct of gene of interest - Ct of ß-actin) vs. time of day (dawn, midday, dusk, and midnight). The lower the mean relative Ct, the higher greater the starting amount of mRNA for the gene of interest.

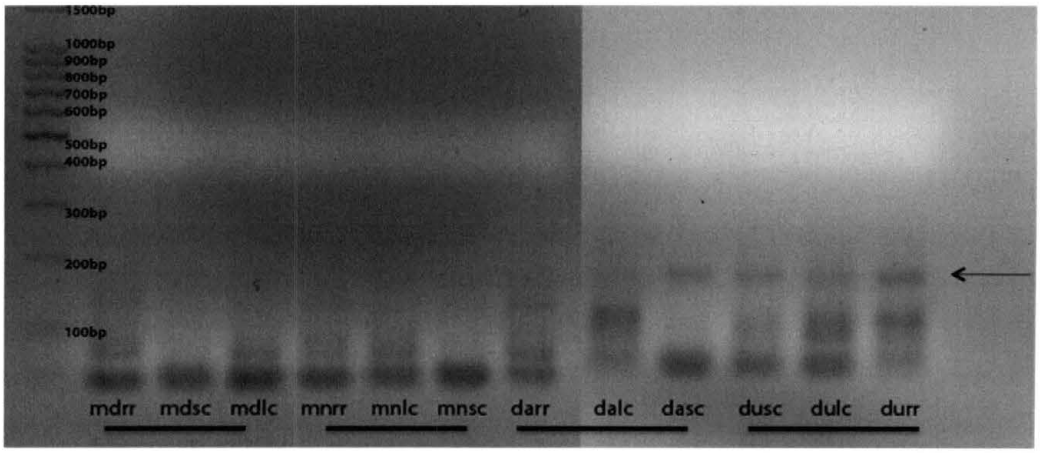

Figure 5. Localization of M_2 receptor to different parts of the eye. A product of the expected size of 140 bp was generated from RNA found in the retina and RPE at all time points. It was also found in RNA generated from the sclera and choroid at dawn and the lens, cornea, sclera, and choroid at dusk.

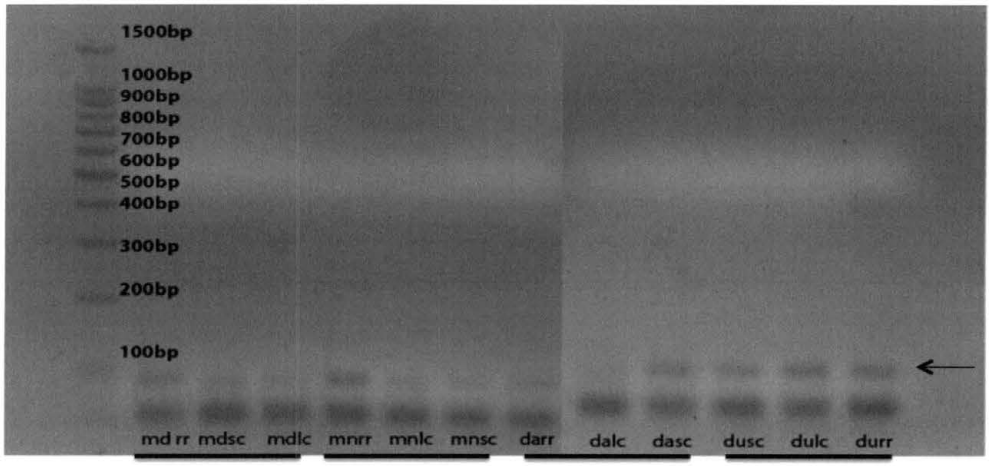

Figure 6. Localization of M_5 receptor to different parts of the eye. A product of the expected size of 88 bp was generated from RNA found in retina and RPE, sclera and choroid and lens and cornea at all time points.

Diurnal Expression of M_3 Receptor Protein

Immunohistochemistry was performed to localize and quantitate expression of the M_3 receptor with the goal of identifying a diurnal pattern of expression. M_3 receptor protein expression was found to differ among the four time points with the most intense labeling at midnight (Figure 10). The labeling at midnight was evident in all layers of the retina. Retinas taken at dawn had little to no labeling while at midday there was some labeling in the retinal ganglion cells and nerve fiber layer (Figures 7 and 8). Retinas collected at dusk had a robust increase in labeling in comparison to dawn and midday, and the protein was evident in most layers of the retina (Figure 9).

Western blot analysis was performed to corroborate immunohistochemistry and to quantitate levels of protein expression. The results indicate diurnal protein expression for the M_3 receptor. Protein extracts from midnight and dawn samples showed labeled bands at 75 kDa and 25 kDa, representing a protein of the expected molecular weight and possibly a degradation product for the M_3 receptor or cross-reactivity with the antibody, respectively. Protein extracts from dusk yielded light bands at the previously mentioned molecular weights and labeled bands were absent from midday samples (Figure 11).

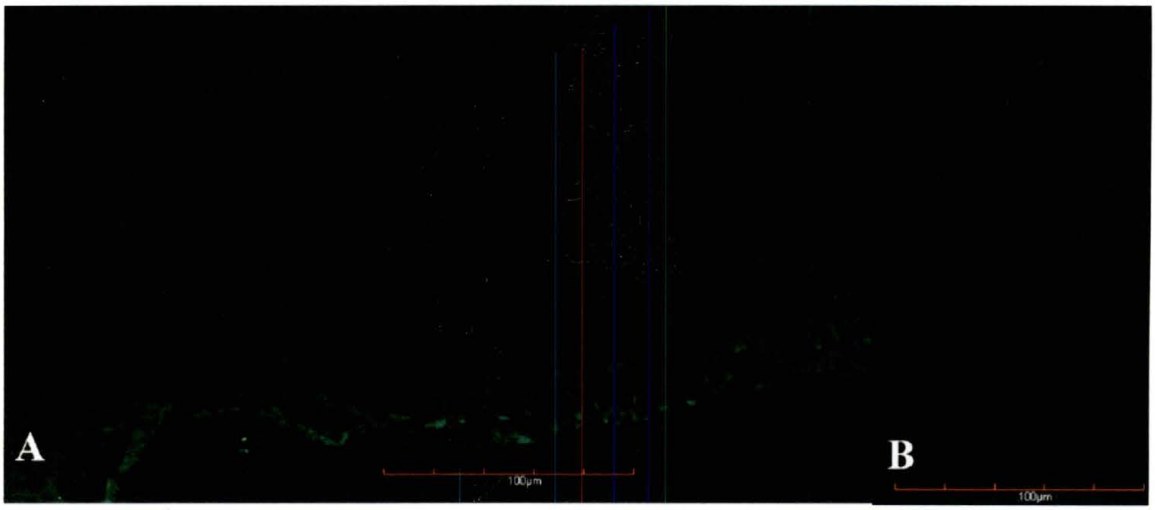

Figure 7. Immunolabeling of M_3 receptor at dawn. A) M_3immunolabeling on section of retina collected at midnight. The image is a projection of 17 optical slices at 1μm thickness. Labeling is evident in al layers of the retina and is more intense than other time points. B) Control retina showing no labeling.

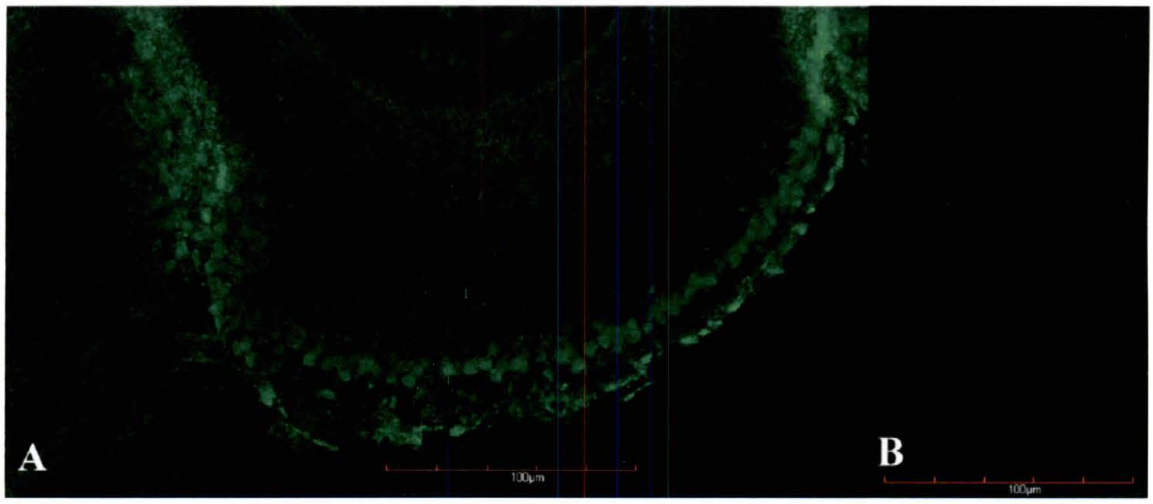

Figure 8. Immunolabeling of M_3 receptor at midday. A) M_3immunolabeling on section of retina collected at midday. The image is a projection of 17 optical slices at 1μm thickness. Labeling is evident in the retinal ganglion cell layer. B) Control retina collected at midday showing no labeling.

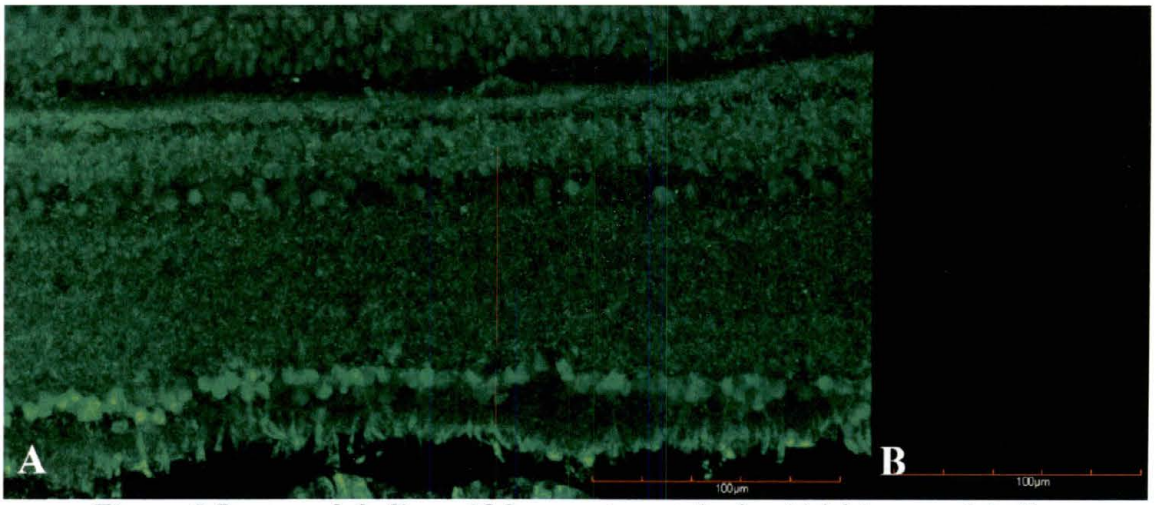

Figure 9.Immunolabeling of M_3 receptor at dusk. A) M_3immunolabeling on section of retina collected at dusk. The image is a projection of 17 optical slices at 1µm thickness. Labeling is evident in all layers of the retina. B) Control retina collected at dusk showing no labeling.

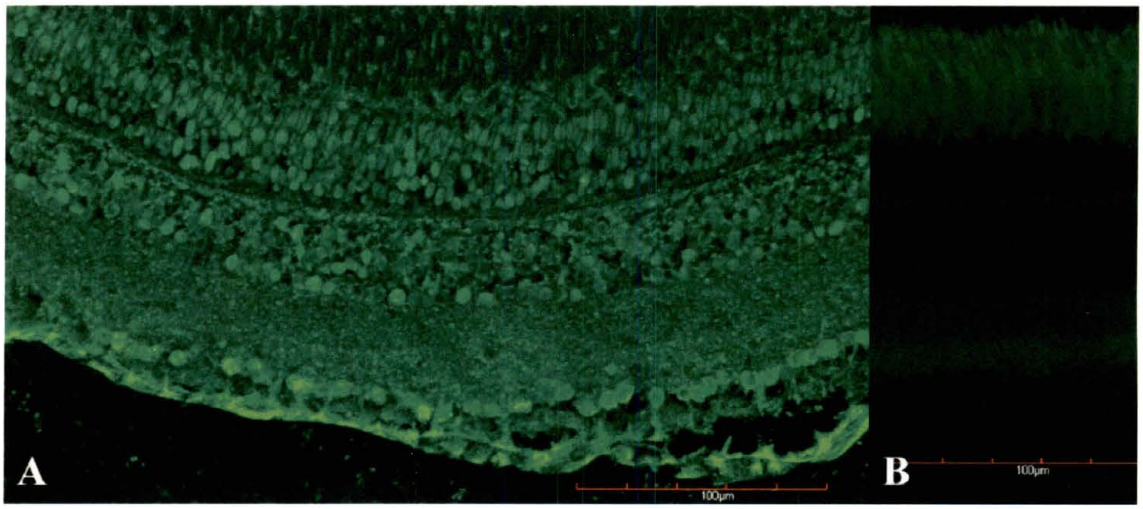

Figure 10. Immunolabeling of M_3 receptor at midnight. A) M_3immunolabeling on section of retina collected at midnight. The image is a projection of 17 optical slices at 1µm thickness. Labeling is evident in all layers of the retina and is more intense than other time points. B) Control retina collected at midnight showing no labeling.

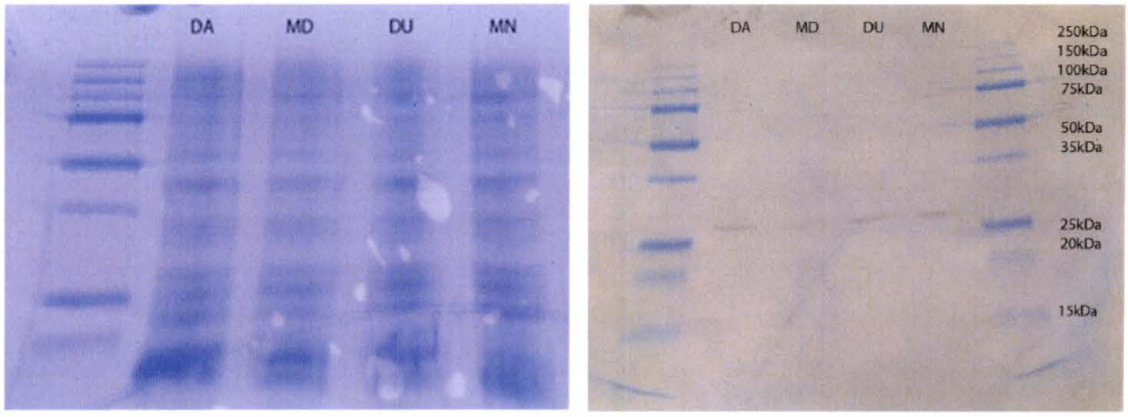

Figure 11. M_3 western blot analysis. Immunoblot using polyclonal anti M_3 antibody at the four time points: dawn, midday, dusk, and midnight. Lanes are labeled as the following: DA=dawn, MD=midday, DU=dusk, and MN=midnight. A) Sister gel stained with Coomassie Blue. B) Nitrocellulose of transferred sister gel labeled with a secondary antibody conjugated to alkaline phosphatase.

Diurnal Expression of Cone Arrestin

Arrestin mRNA was analyzed using qRT-PCR to identify a diurnal pattern of expression. Arrestin mRNA was most highly expressed at dawn and midday (Figure 12). Its expression was also localized to different parts of the eye using RT-PCR and found in all parts (Figure 13).

Immunohistochemistry was used to identify a diurnal pattern of expression for cone arrestin protein. At dawn some labeling is evident in the retinal ganglion cell layer and nerve fiber layer (Figure 14) while at midday labeling is only in the retinal ganglion cell layer (Figure 15). Retinas collected at dusk had the most intense labeling in comparison to all time points. Labeling was evident in the inner plexiform layer, retinal ganglion cell layer, and nerve fiber layer (Figure 16). No labeling was evident in samples collected at midnight (Figure 17) and no labeling was evident in the photoreceptor layer at any time point.

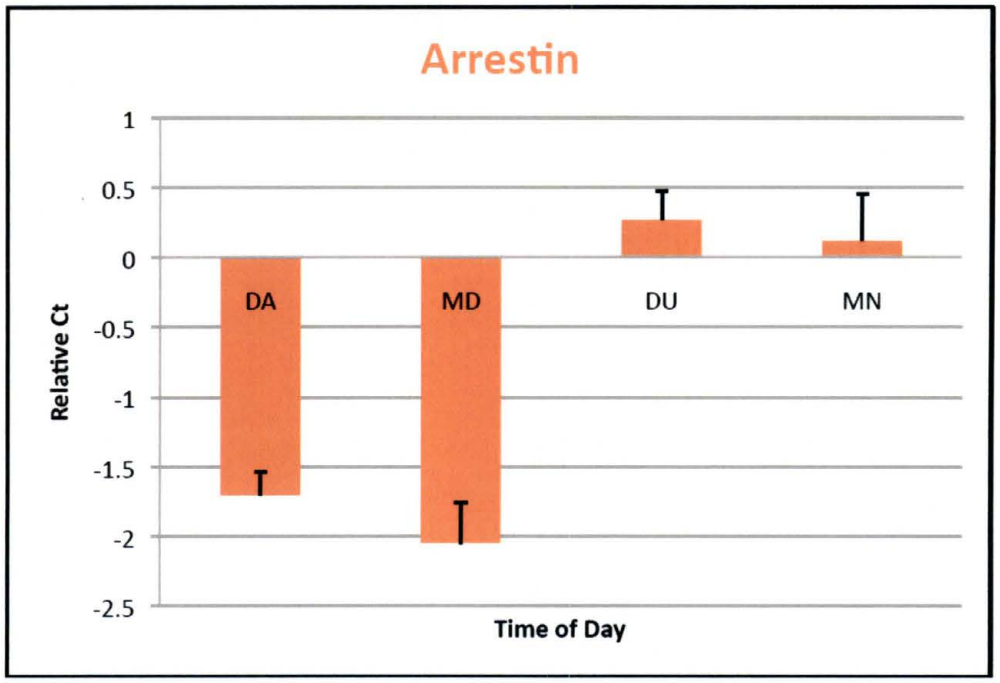

Figure 12. qRT-PCR results: Arrestin. Eyes were collected at the four time points: dawn (DA), midday (MD), dusk (DU), and midnight (MN). RNA was extracted and subsequently subjected to cDNA synthesis and qRT-PCR. The bar graph shows the relative Ct values (Ct of gene of interest - Ct of ß-actin) vs. time of day (dawn, midday, dusk, and midnight). The lower the mean relative Ct, the higher the expression. Bars with a value less than 0 indicate that ß-actin mRNA levels are higher than the levels of mRNA in the gene of interest, and since ß-actin mRNA levels were constant throughout the diurnal cycle, they also indicate high levels of expression of the mRNA of the gene of interest.

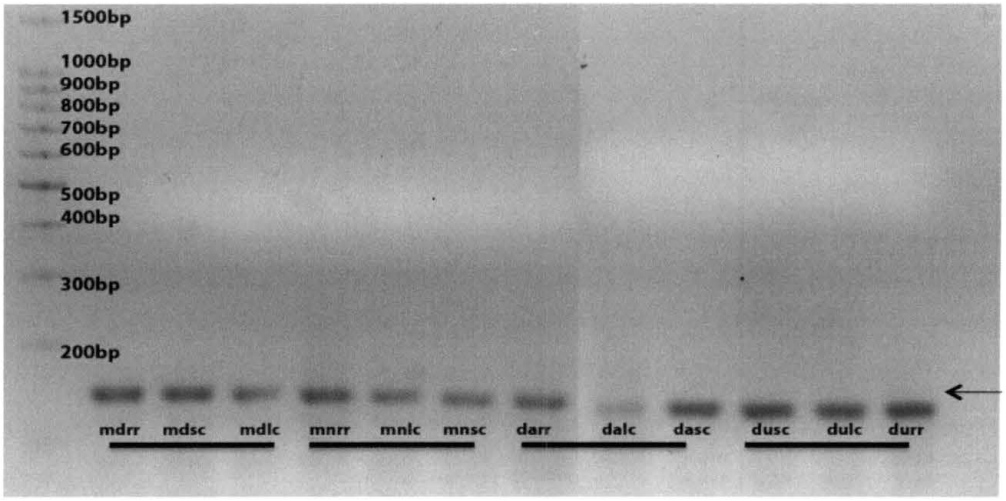

Figure 13. Localization of arrestin (Arr-3) to different parts of the eye. A product of the expected size of 143bp was generated from RNA found in retina and RPE, sclera and choroid and lens and cornea at all time points.

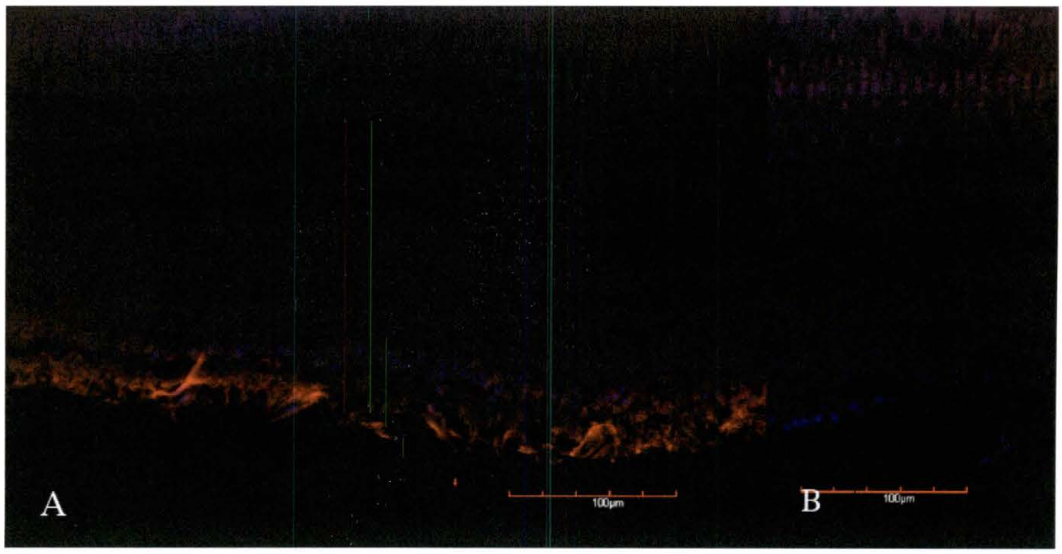

Figure 14. Immunolabeling of cone arrestin at dawn. A) Cone arrestin immunolabeling on section of retina collected at dawn. The image is an optical slice 1µm thick. Blue staining is DAPI indicating the presence of nuclei. Labeling is evident in the nerve finer layer and retinal ganglion cell layer. B) Control retina collected at dawn showing no labeling.

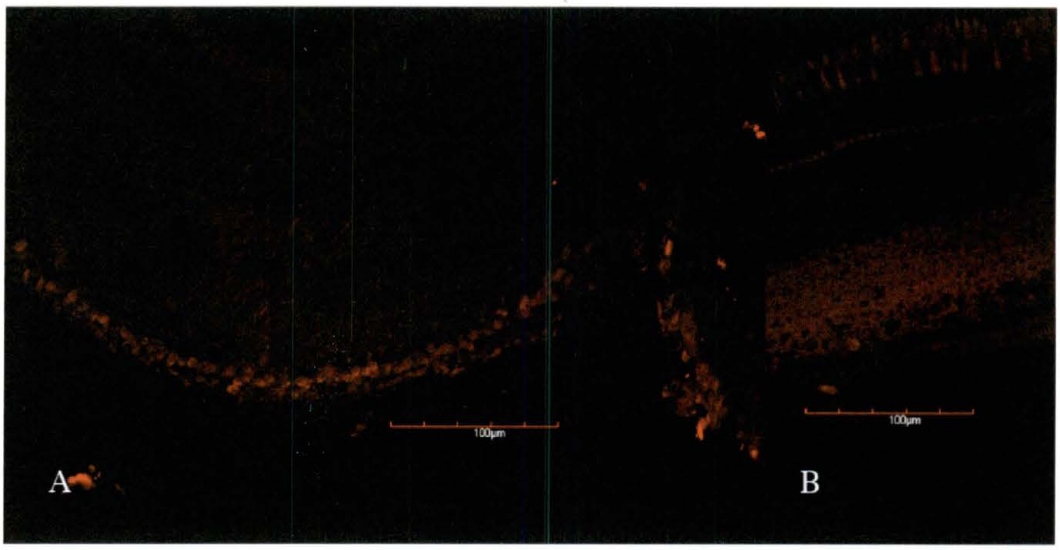

Figure 15. Immunolabeling of cone arrestin at midday. A) Cone arrestin immunolabeling on section of retina collected at midday. The image is an optical slice 1µm thick. Labeling is evident in the retinal ganglion cell layer of the experimental section. B) Control section of retina collected at midday showing no labeling.

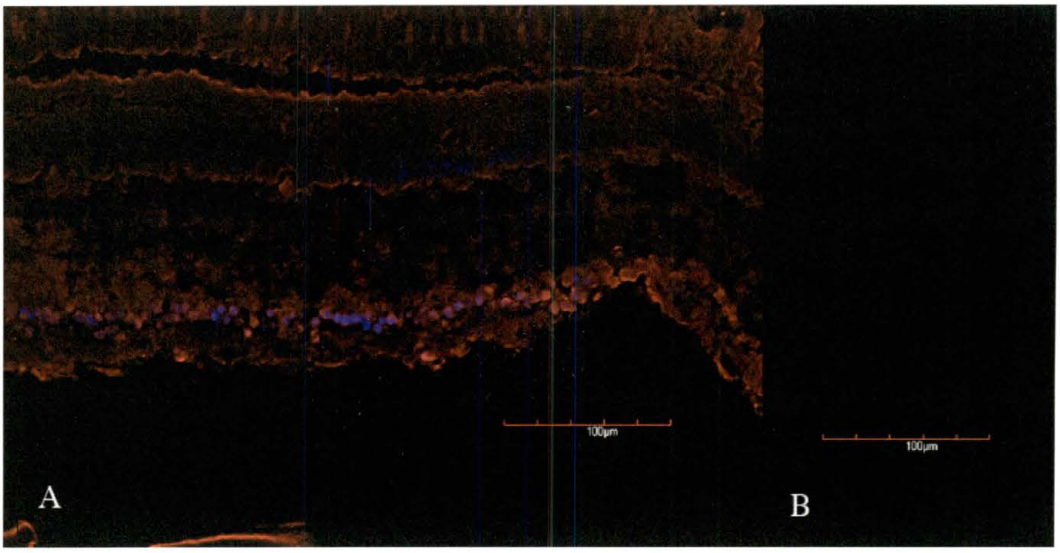

Figure 16. Immunolabeling of cone arrestin at dusk. A) Cone arrestin immunolabeling on section of retina collected at dusk. The image is an opticalslice1µm thick. Blue staining is DAPI indicating the presence of nuclei. Labeling is evident in al layers of the retina and is more intense than other time points. B) Control section of retina collected at dusk showing no labeling.

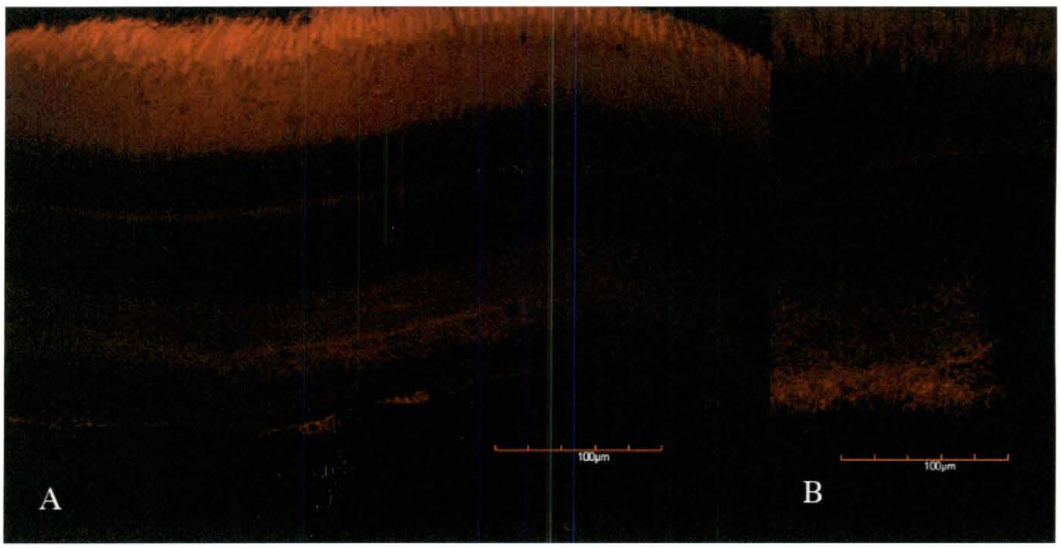

Figure 17. Immunolabeling of cone arrestin at midnight. A) Cone arrestin immunolabeling on section of retina collected at midnight. The image is an optical slice 1µm thick. B) Control section of retina collected at midnight. The control section is not different from the experimental.

CHAPTER IV

DISCUSSION

The purpose of this project was to identify diurnal patterns of gene expression in the eye of zebrafish for the muscarinic and dopaminergic receptors as well as arrestin, a GPCR regulator. The qRT-PCR results showed that for the muscarinic and dopamine receptors analyzed almost all mRNA that was significantly differentially regulated reached its peak at midnight with the exception of D4C. Arrestin mRNA was found to be at its highest levels at midday and dawn. Results from RT-PCR, performed with the goal of mRNA determining in which parts of the eye the various receptors were expressed, indicated the retina and RPE expressed every mRNA tested at all time points. For other parts of the eye, expression depended upon time of day. D4A was expressed by the sclera and choroid at midday and dawn and the lens and cornea at dusk. M_2 was expressed by the sclera and choroid at dawn and dusk as well as lens and cornea at dusk. D4C, M_5, and arrestin mRNA were expressed throughout the eye at all time points analyzed. Additionally, a diurnal pattern of expression for arrestin and M_3 was identified using IHC. The most intense labeling for arrestin was seen at dusk, and there was no labeling at midnight. Arrestin labeling was also evident at dawn and midday in the nerve fiber layer and retinal ganglion cell layer. Immunolabeling of the M_3 receptor was most intense at midnight, and this labeling was localized to all layers of the retina. From the

results of my study, I conclude that in some cases receptor mRNA expression is increased before translation and insertion into the membrane while in other cases mRNA expression may be stably expressed but the resulting protein is differentially expressed

Several studies have been conducted to correlate mRNA expression with protein expression. Pascal et al. (2008) conducted a study of cluster designation genes in cell types of prostate cancer using microarray technology and immunohistochemistry. Their overall goal was to correlate mRNA expression of a given gene with its expression at the protein level. They found that, depending upon the cell type, mRNA expression correlated either positively, moderately positively, or negatively to protein expression. It is thought that a negative correlation is due to error in techniques, sampling methods or an actual biological difference. Their study suggests that an understanding of mRNA expression and protein expression requires identification of the cell type. Ihmann et al. (2004) conducted a study to correlate mRNA expression using qRT-PCR and corresponding protein expression using immunohistochemistry in colorectal carcinoma on pki67 (a cell proliferative marker). Although this study found that there was no significant correlation between immunohistochemical results and qRT-PCR, they did find that high mRNA expression of pki67 was a good indicator of prognosis (Ihmann et al. 2004). In contrast, studies of annexin IV in cancer led the authors to conclude that mRNA expression is correlated to protein expression. Also, mRNA expression within a cell can give a good indication of the cell's state. For example, cancer cells and normal cells can be distinguished from each other using mRNA expression profiles (Zhang et al. 1997). Taken together, the results of these studies suggest that the biological significance of correlation between mRNA and protein expression should be looked at globally within

a cell type and what it means physiologically to the tissue or organism as a whole. They do not suggest that a study of mRNA expression cannot be used to understand protein expression; however, they suggest (along with my own results) that drawing inferences about protein levels or activity from mRNA levels should be done with caution.

Dopamine, as previously stated, acts as a light signal for the retina by inducing light-adaptive retinomotor movements (Dearry and Burnside 1986a). The results of my study show that D2A and D4A receptor mRNA, but not other dopaminergic receptor mRNA, follows a diurnal pattern of expression, suggesting that these dopamine receptors may play a role in the diurnal process of light adaptation. In addition, expression of the dopamine receptors mRNA at their respective time points and expression by the retina and RPE may indicate that one or all of these receptors is also involved in entrainment of the circadian clock by melanopsin or interaction with melatonin. It has been shown that dopamine acting via a D2-like receptor plays a role in melanopsin regulation. Melanopsin is a GPCR that is expressed in a subset of retinal ganglion cells that project to the SCN in rat (Hannibal et al. 2002), and its proposed function is in the entrainment of circadian rhythms (Provencio, et al. 2000; Gooley et al. 2003). Sakamoto et al. (2005), found that a D2-like receptor controls the levels of melanopsin mRNA in a circadian fashion with peak levels of melanopsin mRNA expression found at dusk. Presuming mRNA levels are increased in preparation for translation and insertion in to the membrane, based upon the qRT-PCR data D4C receptor may be involved in the regulation of melanopsin. Since dopamine receptor mRNA was also found to be highly expressed at midnight these receptors could be involved in the interplay between dopamine and melatonin. These findings may be physiologically relevant because

melatonin is known to cause dark-adaptive pigment migration in *Xenopus* (Pierce and Besharse, 1986) while dopamine causes light adaptive pigment migration (Dearry and Burnside, 1986a), and there is a negative feedback loop that exists between dopamine and melatonin. It has been shown that melatonin receptors are localized on the dopaminergic interplexiform cells where they inhibit the release of dopamine, and dopamine receptors are located on photoreceptor cells where they inhibit the synthesis of melatonin (Nguyen-Legros et al., 1996). Although melatonin has been shown to not cause dark adaptive pigment migration in green sunfish (Dearry and Burnside, 1986a; García and Burnside, 1994), this feedback loop may exist in zebrafish between the photoreceptors and interplexiform cells, and it is possible that the interplay between melatonin and dopamine is important in other processes within the eye. In addition, there is evidence in rat that a D4 dopamine receptor is localized to the photoreceptor layer at midnight (Klitten et al., 2008), further suggesting the involvement of a D4 type receptor in the melatonin and dopamine feedback loop. My results are consistent with a model in which D4A and/or D2A are localized to the photoreceptors and participate in the dopamine and melatonin feedback loop. Also, since D4C was most highly expressed at midday, it may be localized to the retinal ganglion cell layer where it regulates melanopsin. These diurnal and circadian interactions of dopamine and melatonin as well as acetylcholine, along with the diurnal expression data of the receptors from my study suggest complex control in the eye which functions to keep the organism in sync with its environment.

M_2 and M_5 mRNA reached their peak expression levels at midnight like D4A and D4C mRNA suggesting that all four receptor subtypes are transcribed and translated at similar times. Interestingly, it is also known that melatonin inhibits the release of

acetylcholine in rabbit retina (Mitchell and Redburn, 1991). Based upon this study and the results from my study, it is possible that there is a feedback loop with melatonin and acetylcholine like the one for dopamine and melatonin. This finding may also indicate a complex interaction between acetylcholine, dopamine, and melatonin. The results of my study indicating that M_5 mRNA is highest at midnight and expressed by the retina and RPE could support previous research indicating a M_{odd} receptor that may be responsible for pigment granule dispersion (González et al., 2004; Phatapekar et al., 2005). Other studies in the García laboratory indicate expression of the M_5 and M_3 receptor subtypes in the adult zebrafish eye (Nuckels, 2006). My thesis is the first to report muscarinic receptor mRNA levels in the eye of fish using qRT-PCR. However, studies in human cornea and conjunctiva using qRT-PCR show fluctuations in mRNA amount, depending upon the cell type analyzed (Liu et al., 2007). This result suggests that more studies of single cell types are necessary in order to determine the physiological relevance of muscarinic mRNA levels in zebrafish eye. RT-PCR studies of muscarinic receptor expression in tree shrew eye indicate expression of all five subtypes in iris/ciliary body, retina, choroid, and sclera, with the exception of M_2, which was not expressed in the cornea (McBrien et al., 2009). My study corroborates some of these findings especially with respect to the expression of M_5. It furthers the findings found in tree shrew retina by analyzing the expression at different time points. In conclusion, my results are consistent with a model that M_5 is located on the RPE and regulates light adaptive pigment granule dispersion while M_2 is located on the photoreceptors and inhibits the levels of cAMP also in preparation for light adaptation.

The immunohistochemical results indicate a diurnal pattern of expression for the M_3 receptor in zebrafish retina. Studies conducted in tree shrew show a spatial pattern of labeling for the M_3 receptor similar to what I report here. Tree shrew retina showed labeling in the outer plexiform layer and nerve fiber layer; there was also labeling in processes that extended thorough the inner nuclear layer and terminated in the inner plexiform layer. These processes are thought to belong to Mueller glial cells (McBrien et al., 2009). The results from my study show labeling in the same layers at midnight, but also in other layers and may indicate the global importance of the M_3 receptor within the retina, especially at midnight. The qRT-PCR data did not indicate a diurnal pattern of expression of this receptor mRNA, but since the protein does follow a diurnal pattern of expression, this suggests other avenues of control of expression for this receptor such as post translational modifications. It is possible that the M_3 receptor is more important to the cell, so there is always mRNA available for it to be translated. Perhaps the M_3 receptor functions in pathways that reflect urgent "just in time" needs for the cell, so mRNA has to be always available, so that protein product can be made on an as needed basis.

In mice, cone arrestin binds to S and M opsins after phosphorylation by GRK-7 (Zhu et al., 2003) and plays a role in adapting the photoreceptors to high light levels; however, my study did not indicate expression of cone arrestin in the photoreceptor layer at any time point. Cone arrestin has been shown to be conserved across species (Hisatomi et al., 1997), and the primary antibody used in my study was raised against human cone arrestin, which is 77% identical to zebrafish cone arrestin (over the antigenic region). Nevertheless the possibility remains that the antibody recognizes something

other than cone arrestin. Alternatively, through the course of evolution, it is possible that cone arrestin has become more specialized in mammals, while in zebrafish it has evolved a wider range of functions or is expressed by a wider range of cell types. One function may be to attenuate signal of GPCRs located in intrinsically photosensitive retinal ganglion cells, which transmit signal to an SCN-like region of the brain for entrainment of circadian rhythms (Gooley et al., 2003).

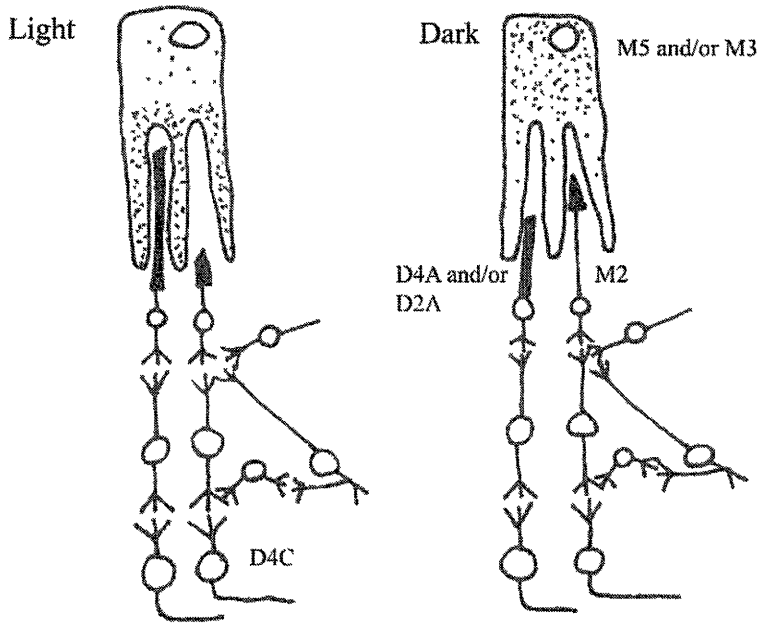

Figure 18. Overall model proposed from results of this research. As discussed above, it is possible that the M_5 and or M_3 receptor is found in the RPE. The M_2 receptor may be found in the photoreceptors as well as D4A and/or D2A and D4C may be located in the retinal ganglion cell layer.

Future Studies

This study provides a starting point for further studies into the relationship between mRNA and protein expression and what the similarities or differences between

the two types of expression mean biologically. Further studies should include an analysis of all genes at the protein level preferably using immunohistochemistry to determine expression level as well as localization within the retina. The importance of mRNA and protein expression of the genes studied should also be observed functionally, for example using gene knockdown or morpholinos. Further studies may also include a dissection of the proposed pathway for the receptor to further illuminate the biological and functional relevance.

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APPENDIX

qRT-PCR primer sequences. Forward and reverse primer sequences with corresponding melting temperatures for all genes analyzed.

Gene	Forward Primer	Tm (°C)	Reverse Primer	Tm (°C)
Arrestin	CGT CGT CCT GTA CTC AGC TGA	58 4	TGA GGC CAG ATT GGT GTC TTC	57 1
Muscarinic Receptor, M1	CAC GCA AGA CAA ACA AGC GGA AGA	60 1	CGC AGA AAG CAT TCA GAA GCA CCA	60 2
Muscarinic Receptor, M2	ATG ATG GTG CTC TAC TGG CAG GTT	60 3	TTG TTG GTG GGT TTG TTG GCT GAG	60 3
Muscarinic Receptor, M3	CTG ATG AAG ACG AGA GCG CAC	58 0	CAG AGC GTG AGA TGC TGC CAT	60 0
Muscarinic Receptor, M5	TGG GTT ACT GGC TGT GCT ATG TCA	60 2	ATG CGG AAG GTC TTC TGG AAG GTT	60 4
Dopamine Receptor, D2A	ACG ATG CTC TCT GTG TGA TTG CGA	60 5	CGT TTG GTG TTG ACA CGT TTC CGT	60 3
Dopamine Receptor, D2B	AAT TAC CTC ATC GTG TCC CTG GCT	60 1	TGA ACC TCC ACT CAC CAA CAA CCT	60 3
Dopamine Receptor, D2C	GGG TGC TTT CAT TCG CCA TTT CCT	60 3	TGA AGG GCA CGT AGA ACG ACA TGA	60 1
Dopamine Receptor, D3	TAG CAG TGG TGA ATG GCT CTG GAA	60 2	AGC TCT CTC TCG AAG CAC AGC AAT	60 2
Dopamine Receptor, D4A	ATG GAT GTC ATG CTT TGC ACT GCC	60 5	ACG TTG TTG ATG CCG AAC ATC ACG	60 2
Dopamine Receptor, D4B	ATG TGA CGC CCA GTA TAG ACC CAA	59 8	ATA AGG ATG AGA GGC ACG CCA CAT	60 3
Dopamine Receptor, D4C	CGT GAG TTT GGC TGT GGC AGA TTT	60 2	CAC ACT GAT TGC GCA CAG GTT GAA	60 3

qRT-PCR results: Genes not statistically significantly regulated. Data is presented as relative Ct for the time point analyzed with n=3. The standard error is also reported (± standard error of the mean).

Gene analyzed	Dawn	Midday	Dusk	Midnight
D2C	6.81 ± 0.1084	6.95 ± 0.1660	7.06 ± 0.0260	6.19 ± 0.4823
D3	8.52 ± 0.2539	9.49 ± 0.5275	N/A	8.41 ± 0.2112
D4B	7.64 ± 0.2681	8.65 ± 0.3219	8.05 ± 1.1852	8.40 ± 0.3537
M_1	9.34 ± 0.2386	9.90 ± 0.3531	12.05 ± 1.3250	8.85 ± 0.3574
M_3	7.14 ± 0.0809	8.40 ± 0.5711	7.21 ± 0.8486	7.39 ± 0.1093

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VITA

Elizabeth Louise Capalbo was born to Janet Lynne Watkins and Andrew Steven Capalbo on February 2, 1984. She was born in Danbury, Connecticut and moved to Houston, Texas at the age of 9. She attended and graduated from Cypress Creek High School in Houston, Texas. After high school graduation Elizabeth attended community college in Houston before moving to Austin, Texas. In 2004, she entered Texas State University-San Marcos as a Biology major. Two years later her son, Payton Wayne Pitts, was born, and in December 2007, Elizabeth graduated with a Bachelor of Science with a major in Biology. She immediately entered into the Masters program at Texas State and has been following her passion for science ever since. She is now engaged to be married and residing in San Marcos, TX. Elizabeth's research focus has been in the area of diurnal and circadian rhythms and the function of these rhythms in the eye. In the future she would like to research further into the workings of the brain in hopes of understanding complex processes addiction and mental disorders, such as Alzheimer's.

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